—Review—

Calcium Release at Fertilization: Artificially Mimicking the Oocyte's Response to Sperm

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Abstract. The mechanism of sperm-induced calcium release has been the subject of many studies since the development in the late 1950s of *in vitro* culture systems that support mammalian fertilization. Despite efforts to elucidate the nature of the signal from the sperm that triggers both the early and late events of oocyte activation, the precise mechanism remains unresolved. Now, with the advent of somatic nuclear transfer technologies, the need to better understand this unique process has been recognised. Nuclear transfer embryos must be induced to commence development artificially because the activating signal from the sperm is absent. The primary activating stimulus is a large increase in the concentration of intracellular-free calcium and numerous physical and chemical treatments have been found to induce calcium changes that initiate the events of oocyte activation. Although live cloned offspring have been produced in a number of species, the overall efficiencies of the nuclear transfer procedures described thus far are unacceptably low and phenotypic anomalies are common. With the aim of improving these efficiencies, researchers are developing artificial activation treatments which induce oocyte responses that mimic those induced by fertilizing sperm. One strategy is to replicate the pattern of calcium change more closely. Another strategy is to couple an activating stimulus with treatments that inhibit maturation (or M-phase) promoting factor (MPF) activity, which regulates meiotic progression in oocytes. This paper reviews what is understood of calcium release at fertilization and describes the treatments that have been used to induce oocyte activation artificially in parthenogenetic and nuclear transfer studies. The relative effectiveness of the strategies employed to mimic the oocyte's response to sperm are discussed.

Key words: Oocyte activation, Fertilization, Parthenogenesis, Nuclear transfer

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To produce a viable embryo at fertilization, the sperm not only provides the male chromosomal complement, but also the stimulus that initiates development. The mechanism by which the activating signal is transmitted from the sperm to the oocyte is not fully clarified, but it is clear that this signal triggers a large increase in the concentration of intracellular-free calcium. In fertilized sea urchin and *Xenopus* eggs, the calcium

increase takes the form of a propagating wave that originates at the point of sperm attachment and traverses the egg at a velocity of 5 to 10 $\mu\text{m}/\text{sec}$ [1]. Studies in the hamster [2], mouse [3], cow [4] and pig [5] demonstrate that mammalian oocytes display a similar increase of intracellular calcium at fertilization, but that the initial transient rise is followed by a series of smaller transient increases. The frequency of the calcium transient increases, or oscillations, varies among species, occurring at intervals of 1 to 10 min in hamster oocytes [4] and

15 to 51 min in cattle oocytes [2]. These patterns of sperm-induced calcium oscillations can continue in mammalian oocytes for several hours after fertilization [1]. The precise role of the additional calcium spikes during mammalian fertilization remains unclear. Some evidence suggests that the multiple calcium rises may be involved in the regulation of later developmental events, such as compaction and blastocyst formation [6–9].

The ability of the oocyte to release calcium in response to the fertilizing sperm increases during oogenesis [10]. In mature mouse oocytes, the first sperm-induced calcium transient increase is much larger in amplitude and longer in duration than in immature oocytes [11]. In addition, fertilization of immature mouse oocytes only generates two or three calcium oscillations, which cease within 1 h, compared with the highly repetitive series of calcium oscillations lasting 2 to 3 h in mature oocytes [12]. The modifications in the calcium release response during oocyte maturation may be due to an increase in the size of the internal calcium store or an increase in the density of the calcium channels [10]. There is evidence that the cell cycle plays an important role in the acquisition of these changes and that the oocyte must be in the metaphase stage of the cell cycle at fertilization in order to generate a normal calcium release response [13].

Calcium Release Mechanisms

The mechanism(s) by which calcium release is mediated at fertilization has been the subject of many studies. Calcium release mechanisms that have been found to exist in mammalian oocytes include those that are regulated by inositol trisphosphate receptors, ryanodine receptors and calcium itself.

Inositol trisphosphate receptors

Many cellular processes, including cell growth, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signalling, result in an increase of intracellular calcium [14]. In the majority of these systems, external stimuli, such as neurotransmitters, hormones and growth factors, interact with cell surface receptors that are coupled to mechanisms which activate phospholipase C (PLC) to hydrolyze

an inositol lipid precursor stored in the plasma membrane. The hydrolysis of this lipid precursor, phosphatidylinositol 4,5-bisphosphate (PIP₂), releases inositol 1,4,5-trisphosphate (InsP₃) into the cytoplasm which then binds to InsP₃ receptors (InsP₃Rs) (see Fig. 1). The InsP₃R functions as a major intracellular calcium channel and is normally located in portions of the endoplasmic reticulum (ER). Upon binding InsP₃, the receptor undergoes a conformational change which opens the channel, thereby mobilizing calcium from internal stores [14]. The InsP₃Rs are abundant in mammalian oocytes and have been shown to mediate calcium release during maturation in mouse [10], hamster [15] and cattle oocytes [16] and to generate calcium oscillations during fertilization in hamster [17] and cattle oocytes [4]. Furthermore, ooplasmic redistribution and an increase in the amount of this receptor during maturation have been correlated with an increased ability to release calcium at the metaphase-II (M-II) stage [16, 18, 19].

Ryanodine receptors

In muscle cells, calcium release is predominantly mediated by ryanodine receptors (RyRs), which are primarily associated with the sarcoplasmic reticulum (SR) [14]. The RyR mobilizes calcium from internal stores in response to the plant alkaloid ryanodine and reagents such as caffeine, cyclic adenosine diphosphate ribose (cADPR) and fatty acid derivatives [20]. Although the RyR is almost twice as large as the InsP₃R they share considerable structural and functional similarities [21]. Both receptors are tetrameric, with the membrane-spanning C-terminal regions of each monomer combining to form the calcium channel, and the large N-terminal domains presenting the binding sites to the cytosol [14]. The remarkable structural similarities and the regions of sequence homology that exist between the RyRs and InsP₃Rs suggest they have a common evolutionary origin. Like the InsP₃Rs, RyRs are present in mammalian oocytes and have been shown to mediate calcium release in mouse [12, 22], cattle [23, 24], pig [25] and human [26] oocytes.

Even though the existence of both InsP₃Rs and RyRs in mammalian oocytes has been established [23, 25, 27], several lines of evidence suggest that the release of calcium at fertilization is most likely induced by InsP₃ [10]. Firstly, sperm-induced calcium oscillations were completely blocked in

hamster oocytes injected with a monoclonal antibody to the InsP_3Rs [28]. Secondly, mouse oocytes injected with a monoclonal antibody that binds to the InsP_3Rs failed to exhibit both early and late events of oocyte activation following insemination [29]. Thirdly, the density of the RyRs is reported to be as much as 100-fold lower than that of the InsP_3Rs in cattle oocytes [16]. Finally, in mouse oocytes, expression of the type I isoform of the InsP_3R protein was found to increase during oocyte maturation and decrease dramatically within 8 h of fertilization [30]. The contribution of the RyRs to calcium release at fertilization, if any, has not yet been established.

Calcium-induced calcium release

The repetitive pattern of calcium transient increases observed in mammalian oocytes in response to fertilizing sperm is also displayed in many of the cells that respond to calcium-mobilizing stimuli [14]. A regenerative process of calcium-induced calcium release (CICR) drives the generation of each calcium spike [21]. Another functional property shared by the RyRs and the InsP_3Rs , which is responsible for both the spatial and temporal patterns of calcium signalling, is their variation in sensitivity in response to intracellular-free calcium [14]. A generalized CICR model of calcium spiking proposes that when internal calcium stores carrying RyRs or InsP_3Rs are charged with calcium, the sensitivity of the receptors is increased. Calcium channels nearest to the site of the external stimulus open in response to a relatively small increase in agonist levels, releasing calcium into the cytosol. This localized calcium increase mediates a progressive release of calcium from neighbouring stores by the CICR process, propagating a "wave" of calcium increase throughout the cell. Once the cytosolic calcium reaches a threshold level it then inhibits further calcium release by exerting a negative feedback effect on the calcium channels. Calcium is then pumped out of the cytoplasm by active transport and the internal calcium stores are recharged. An influx of extracellular calcium, through calcium channels located in the plasma membrane, is also required to help replenish the internal stores of calcium.

Transduction of the Activating Signal

At fertilization of the mammalian oocyte, the sperm first binds to and then fuses with the oocyte plasma membrane. The interaction between the gamete membranes activates specific biochemical pathways within the oocyte, which triggers the release of calcium. There has been a great deal of debate as to whether fusion of the gamete plasma membranes is a prerequisite for oocyte activation, or whether binding of the sperm to cell surface receptors can initiate oocyte activation. Consequently, the two hypotheses that have been proposed to explain how the activating signal is transmitted from the sperm to the oocyte are based on cell surface receptor-mediated signalling pathways and cytosolic sperm factor-mediated signalling pathways (see Fig. 1).

Receptor-mediated signalling pathways

At fertilization, an increase in InsP_3 may be triggered by the sperm binding to surface receptors in the oocyte plasma membrane. There are two major receptor-mediated pathways that activate PLCs by separate transducing mechanisms to stimulate the formation of InsP_3 . One pathway is initiated by receptors that are coupled to G proteins (or guanine nucleotide-binding proteins) and the other by receptors that activate tyrosine kinases directly or indirectly [14].

Members of the G protein family transduce signals from receptors at the cell surface to internal cellular components by activating PLC- $\beta 1$, a PLC isozyme [14]. The activation of PLC- $\beta 1$ by G proteins requires energy in the form of guanosine triphosphate (GTP), which is hydrolyzed to guanosine diphosphate (GDP) in the process [14]. The theory that sperm-induced calcium release is mediated by G protein-linked receptors is supported by several studies that have examined the effects of G protein activators and inhibitors on oocytes. For example, injection of the metabolically stable GDP analogue, guanosine-5'-O-(2-thiodiphosphate) ($\text{GDP}\gamma\text{S}$), inhibited intracellular calcium oscillations in fertilized hamster [31] and rabbit [32] oocytes and blocked G protein-mediated calcium release in porcine oocytes [33]. Conversely, injection of guanosine-5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$), a hydrolysis-resistant analogue of GTP, caused repetitive intracellular calcium increases in unfertilized hamster [31, 34]

Fig. 1. The proposed mechanisms of sperm-induced calcium release in mammalian oocytes include receptor-mediated and sperm factor-mediated signalling pathways. Sperm (A, B) binds to a receptor (R) that is linked to either a G protein (G) or tyrosine kinase (TK), which activates (+) phospholipase C (PLC). Alternatively, sperm (C) fuses with the oocyte plasma membrane and releases a sperm factor into the cytoplasm that may stimulate, or have, PLC activity (?). A component of the plasma membrane, phosphatidylinositol 4,5-bisphosphate (PIP₂), is hydrolysed by PLC to inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). The second messenger InsP₃ then binds to its receptor (InsP₃R), releasing calcium from internal stores and increasing the concentration of intracellular-free calcium ($\uparrow[Ca^{2+}]_i$). This calcium increase induces cellular events that are critical to the activation process. The ryanodine receptor (RyR) may mediate calcium-induced calcium release.

and rabbit [32] oocytes. Treatment with serotonin and carbachol, specific activators of G proteins, also triggered calcium oscillations in hamster [34] and mouse [22] oocytes.

Later events associated with fertilization, such as cortical granule exocytosis, pronuclear formation and embryo development to the blastocyst stage,

were evoked in porcine oocytes injected with GTP γ S [35]. In contrast, pronuclear formation and cleavage to the 2-cell embryo stage were inhibited in fertilized mouse oocytes injected with G protein inhibitors [36]. Finally, in oocytes expressing the G protein-coupled muscarinic receptor, treatment with acetylcholine, the receptor's agonist, stimulated a variety of fertilization-related events including pronuclear formation [37]. Together, these findings clearly demonstrate that the G protein signal transducing mechanism exists in mammalian oocytes, but direct involvement of this pathway at fertilization has not been shown.

The signalling pathway could be mediated by sperm binding to receptors that are instead coupled to tyrosine kinases. Tyrosine kinases can generate InsP₃ by activating PLC- γ 1, another PLC family member, in a phosphorylation process that requires adenosine triphosphate (ATP) [14]. Growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), engage two receptors, bringing them together to form a dimer. Each receptor contains a cytoplasmic tyrosine kinase domain that phosphorylates the other on specific tyrosine residues, thus enabling PLC- γ 1 to dock with the receptor and in turn be phosphorylated.

Tyrosine kinase activity has been demonstrated in sea urchin eggs following fertilization [38, 39] and stimulation of the EGF receptor tyrosine kinase activity induces a calcium wave in *Xenopus* eggs [40]. In mouse oocytes, fertilization results in the increased phosphorylation of a set of proteins, predominantly on tyrosine residues [41]. Immunodetection of PLC proteins using monoclonal antibodies reveals that PLC- γ 1, which is activated by tyrosine phosphorylation, is present in mature mouse oocytes and spermatozoa [42]. Finally, a role for tyrosine kinases at fertilization is supported by the finding that preincubation of mouse oocytes with tyrosine kinase inhibitors significantly hinders the normal sperm-induced calcium release response [42].

Integrins are a major family of cell adhesion receptors that have been implicated in the binding and fusion of sperm and oocyte plasma membranes [43]. Several integrins have been detected on the surface of mammalian oocytes [44–47], and characterization of the sperm transmembrane protein fertilin has revealed the presence of a "disintegrin" domain that may bind integrins [48].

Peptide analogues of the disintegrin domain and an antibody against the $\alpha 6$ integrin subunit inhibit sperm-oocyte binding and fusion in the mouse [44, 45]. Studies in many cell types have shown that integrin stimulation can induce tyrosine phosphorylation and increase the intracellular-free calcium concentration [49]. Furthermore, peptides containing the Arg-Gly-Asp (RGD) sequence, which is known to bind integrins, induced calcium release, cortical granule exocytosis and resumption of meiosis in *Xenopus* eggs [50].

Sperm factor-mediated signalling pathway

Although the evidence in support of a receptor-mediated signalling pathway is persuasive, a number of findings suggest that the signal transduction mechanism which triggers calcium release in response to sperm binding operates through a sperm factor-mediated signalling pathway. This mechanism requires the oocyte and sperm plasma membranes to fuse, and for an aqueous pore between the gamete cytoplasm to form that allows a cytosolic factor to diffuse from the sperm to the oocyte. Upon entering the oocyte, the sperm factor is hypothesized to mediate calcium release directly or indirectly. In sea urchin eggs, it has been found that sperm-egg fusion is essential for fertilization to take place, and the formation of an aqueous pore between the egg and sperm cytoplasm has been demonstrated [1]. Further support for this hypothesis comes from the finding that sperm-oocyte fusion precedes the initial intracellular calcium increase in mouse oocytes by 1 to 3 min [51].

The results of experiments in which putative activating messengers have been injected into oocytes indicate that the sperm factor cannot be calcium itself, nor can it be InsP_3 , cADPR or cyclic guanosine monophosphate (cGMP) [1]. At present, the most promising candidate messenger is a protein factor isolated from the cytosol of hamster and boar sperm [52]. Injection of this factor into hamster oocytes has been shown to trigger a series of calcium transient increases identical to those seen at fertilization [52]. Cytosolic sperm extracts also stimulated repetitive calcium oscillations following injection into mouse [53–55], cattle [56] and human [57] oocytes. Interestingly, injection of the same porcine sperm extract induced calcium oscillations of high frequency in mouse oocytes and low frequency in cattle oocytes, similar to the

patterns of calcium release observed in each species at fertilization [56]. Later events associated with fertilization, such as pronuclear formation and cleavage, have also been induced in rabbit [58], mouse [55] and cattle [59] oocytes injected with a protein factor obtained from sperm.

Injection of intact sperm directly into the cytoplasm of human oocytes, or intracytoplasmic sperm injection (ICSI), induces calcium increases after a lag period of 4 to 12 h [60] and results in the birth of normal offspring [61–63]. These results indicate that the fusion of sperm and oocyte plasma membranes can be bypassed, and that the stimulus responsible for activating the oocyte is probably due to a delayed release of a factor from the injected sperm. In the mouse, the amount or activity of this factor appears to increase during spermiogenesis because testicular spermatozoa induced pronuclear formation when injected into oocytes, whereas round spermatids did not [64]. In isolated heads of mature mouse and hamster epididymal spermatozoa, the factor is apparently associated with the perinuclear material [65]. Round spermatids by themselves can not induce calcium release when injected into mature oocytes [66], but can still give rise to normal offspring following ICSI if the oocyte is activated artificially [64, 66]. In addition, the putative activating sperm factor may become associated with the embryonic nuclei, as nuclei transferred from fertilized 1- and 2-cell stage mouse embryos induced intracellular calcium transient increases in unfertilized oocytes [67].

The action of the sperm activating factor is not highly species-specific because mouse oocytes are activated following injection of spermatozoa from the hamster, rabbit, pig, human and even fish [65]. Indeed, sperm of chicken, *Xenopus* and the flowering plant, *Brassica campestris*, contain a cytosolic soluble protein factor that triggers calcium oscillations in mouse oocytes [68, 69]. The specificity of action of the sperm factor in triggering calcium oscillations in mammalian oocytes appears to be dependent upon the presence of maternal machinery. Tang *et al.* [70] found that prior exposure to sperm extract inhibited sperm factor-induced calcium oscillations in mouse oocytes, whereas prior exposure to an artificial calcium-releasing stimulus did not. This finding suggests that the maternal machinery responsible for producing the calcium oscillations at fertilization operates only once and is only inactivated by a

sperm-derived component [70].

In an effort to characterize the active component of sperm, Parrington and coworkers [71] separated cytosolic extracts from boar sperm using three successive chromatographic columns with different separation properties. The fractions that released calcium from sea urchin egg homogenates also induced calcium oscillations following injection into mouse oocytes [71]. The calcium-releasing activity of boar sperm extract was found to be due to the action of a sperm-derived PLC, which hydrolyzed PIP₂ to InsP₃ [72]. Furthermore, recombinant PLCs and PLCs purified from boar brain, liver and kidney failed to release calcium in sea urchin egg homogenates or intact mouse oocytes, indicating that sperm PLC activity has distinct properties [73]. Other findings indicate that the putative sperm factor may generate InsP₃ by stimulating PLC activity indirectly. A truncated form of the c-kit tyrosine kinase receptor, termed tr-kit, has been identified in the cytoplasm of mature mouse spermatozoa [74]. Injection of cellular extracts containing the tr-kit protein into mature mouse oocytes induced cortical granule exocytosis, resumption of meiosis and cleavage [75].

Although the precise nature and site of action of the activating signal at fertilization has not been resolved, the evidence in support of the sperm factor-mediated signalling pathway is compelling. The receptor-mediated signalling pathway that triggers calcium release in many other cellular processes has not been ruled out, but for a process as extraordinary as fertilization, where the sperm provides a unique delivery system for the activating signal, it seems reasonable to propose that a novel sperm factor mediates the response. Of the putative activating agents tested, the cytosolic factor present in sperm extract is the only candidate that can generate a pattern of intracellular calcium oscillations identical to that seen at fertilization [54]. Clearly, further studies are needed to confirm whether (i) the sperm factor-mediated signalling pathway functions in mammalian oocytes at fertilization and (ii) a single sperm contains enough sperm factor to trigger oocyte activation.

The Role of Calcium at Activation

The increase of intracellular calcium following fertilization is recognized as the primary signal

responsible for the initiation of development (reviewed by [76]). This conviction is supported by experiments that either suppress the natural rise of calcium following fertilization, which inhibits oocyte activation, or raise the intracellular-free calcium concentration artificially, which stimulates oocyte activation. Events of oocyte activation that are regulated by calcium include the resumption of meiosis, cortical granule exocytosis, and possibly new protein synthesis [77]. Inhibition of calcium transient increases in fertilized mouse oocytes using the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) blocked cortical granule exocytosis and second polar body formation [3]. Other fertilization-associated events, such as zona pellucida modifications that result in a block to polyspermy, the decrease of histone H1 kinase activity, pronucleus formation, the recruitment of maternal mRNAs and post-translational modifications of proteins, were inhibited in mouse oocytes injected with a monoclonal antibody that binds to the InsP₃Rs and inhibits InsP₃-induced calcium release [29]. Conversely, treatments that increase the concentration of intracellular-free calcium in the absence of sperm induce both early and late events of oocyte activation. The details of these treatments and their effects on mammalian oocyte activation are described later.

A number of studies indicate that the target effectors of calcium that are involved in the regulation of early and late events of oocyte activation include the calcium-dependent protein kinase C (PKC), calmodulin, and the calmodulin-dependent protein kinase II (CaMK_{II}) (reviewed by [77]). In mouse oocytes, activation of PKC has been shown to induce cortical granule exocytosis [78] and initiate the structural changes required for second polar body formation [79]. Evidence also suggests that PKC is involved in regulating sperm-induced calcium oscillations by exerting a negative feedback effect on calcium release [80]. Furthermore, PKC has been implicated in the resumption of meiosis [81], cortical granule exocytosis [82–84] and pronuclear formation [85, 86] in artificially activated oocytes. However, other evidence suggests that a different calcium-dependent pathway, involving calmodulin and CaMK_{II}, is responsible for the resumption of meiosis at fertilization [87]. Calmodulin appears to be involved in the resumption of the cell cycle since

meiotic progression and a decrease in histone H1 kinase activity were inhibited in fertilized mouse oocytes treated with the calmodulin antagonist W-7 [88]. However, W-7 treatment did not block cortical granule exocytosis, indicating a different pathway is involved in this activation-associated event [88]. In the same study, low concentrations of injected free calcium induced cortical granule exocytosis without triggering meiotic resumption, suggesting that the pathways have differing sensitivities to calcium [88]. A role for calmodulin in meiotic resumption is supported in studies of *Xenopus* eggs that found both cytostatic factor and maturation-promoting factor were inactivated by CaMK_{II} at fertilization [89].

Maturation-Promoting Factor

Meiosis is arrested in mature mammalian oocytes at the second metaphase (M-II) stage. Suspension of the oocyte cell cycle is maintained by a heterodimeric protein kinase called maturation (or M-phase) promoting factor (MPF), which is highly conserved [90]. The MPF protein complex consists of a regulatory subunit comprising of cyclin B [91] and a catalytic subunit comprising of a cyclin-dependent protein kinase that is the 34 kDa product of the *cdc2* gene (p34^{cdc2}) [92, 93]. Activation of MPF is induced by cyclin B and requires phosphorylation of threonine residue 161 and dephosphorylation of tyrosine residue 15 (and threonine residue 14 in vertebrates) of the p34^{cdc2} subunit [94, 95].

In eukaryotic cells, the kinase activity of MPF is thought to initiate a series of reactions that result in nuclear envelope breakdown (NEBD), chromosome condensation and assembly of the metaphase spindle [90]. A major substrate of active MPF is the chromosomal packaging protein histone H1 [90]. Phosphorylation of histone H1 by cellular extracts forms the basis of an assay used to determine the level of MPF activity during meiosis and mitosis [90]. Changes in the level of MPF activity have been found to be similar during maturation of mouse [96, 97], rabbit [98], goat [99], cattle [100–102] and pig [103–107] oocytes. Numerous studies have found that the level of MPF activity is relatively low at the germinal vesicle stage and steadily increases as meiosis progresses to the first metaphase (M-I) stage. The level of MPF

activity then decreases markedly at the anaphase-I and telophase-I stages, but increases again by the M-II stage to a level similar to that in M-I oocytes. Accumulation of newly synthesized cyclin B corresponds to an increase in MPF activity, while cyclin B degradation by ubiquitin-dependent proteolysis corresponds to a decrease in MPF activity [107, 108]. In contrast, the level of p34^{cdc2} remains unchanged throughout meiosis [96, 107].

In M-II arrested oocytes, the elevated levels of MPF activity are maintained by a factor known as cytostatic factor (CSF), which prevents cyclin B degradation [90]. CSF activity has been shown to be associated with a number of different proteins including mitogen-activated protein kinase (MAP kinase or MAPK) [109], cyclin-dependent kinase 2 (cdk2) [110] and products of the *raf-1* gene [111] and *c-mos* proto-oncogene [112]. As mentioned previously, CSF is inactivated by CaMK_{II} in response to the sperm-induced intracellular calcium increase at fertilization [89]. Inactivation of CSF facilitates cyclin degradation, which decreases the levels of MPF activity in the oocyte, thereby initiating disassembly of the metaphase spindle [113].

MPF activity has been shown to return to basal levels following insemination in mouse [96], pig [114] and cattle [27] oocytes, within 3, 4 and 5 h respectively. Increasing the intracellular-free calcium concentration of mammalian oocytes artificially, using chemical or physical stimuli, has also been found to reduce the levels of MPF activity, often within one hour of treatment [27, 33, 115–121].

Artificial Activation

In the absence of sperm, the concentration of intracellular-free calcium can be elevated in mammalian oocytes by many different treatments. Some of the treatments that have been used to induce an intracellular calcium increase in mouse, hamster, rabbit, cattle, pig and human oocytes are shown in Table 1. Cytoplasmic injection of solutions containing Ca²⁺, InsP₃R agonists, RyR agonists or G protein activators, or incubation with alcohols, calcium ionophores, Sr²⁺ or thimerosal have all been shown to induce a transient intracellular calcium increase in mammalian oocytes. The infusion of extracellular Ca²⁺ or InsP₃

Table 1. Artificial activation treatments that have been shown to induce intracellular calcium changes in mammalian oocytes

Species	Reagent	Concentration	Application	Reference
Mouse	Ca ²⁺	≤ 0.9 mM	Electric pulse	[123, 129]
	Ca ²⁺	0.5 M	Injection	[53]
	InsP ₃	0.002–1 mM	Injection	[11, 53]
	Adenophostin	2–10 μM	Injection	[66]
	Sr ²⁺	4.6–9.2 mM	Incubation	[3]
	Ethanol	7–8.6%	Incubation	[140, 155]
	Butanol	0.5%	Incubation	[155]
	Acetylcholine	50 μM	Incubation	[153]
	Thapsigargin	10 μM	Incubation	[12]
	Ryanodine	1 mM	Incubation	[12]
	A23187	5–10 μM	Incubation	[3, 140]
	Ionomycin	5–10 μM	Incubation	[11, 12]
	Thimerosal	1–200 μM	Incubation	[11, 153]
Hamster	InsP ₃	0.08–240 μM	Injection	[34, 80]
	GTP	5–10 mM	Injection	[34]
	GTPγS	0.33–20 mM	Injection	[34, 80]
	Thimerosal	200 μM	Incubation	[17]
	Serotonin	50 nM	"Puffing" method	[158]
Rabbit	Ca ²⁺	0.1–1 mM	Electric pulse	[122, 124]
	Ca ²⁺	1.8 mM	Injection	[32]
	InsP ₃	5 μM	Injection	[32, 154]
	InsP ₃ S ₃	500 μM	Injection	[154]
	GTPγS	5–20 mM	Injection	[32]
	Thimerosal	400 μM	Incubation	[154]
Pig	Ca ²⁺	100 μM	Electric pulse	[5]
	Ca ²⁺	0.1 M	Injection	[119]
	InsP ₃	10–250 nM	Injection	[25]
	Ryanodine	0.04–1.2 mM	Injection	[25]
	cADPR	8–200 μM	Injection	[25]
	A23187	50 μM	Incubation	[141, 142]
Cattle	Thimerosal	200 μM	Incubation	[152]
	Ca ²⁺	100 μM	Electric pulse	[6]
	Ethanol	7%	Incubation	[139]
	InsP ₃	0.05–190 μM	Injection	[4, 23, 24, 27, 139, 151]
	InsP ₃ S ₃	500 μM	Injection	[151]
	GTPγS	20 mM	Injection	[151]
	Ryanodine	100–200 μM	Injection	[23, 24, 27]
	Caffeine	10–20 mM	Injection	[23]
	cADPR	4–8 μM	Injection	[23, 27]
	A23187	10 μM	Incubation	[139]
	Ionomycin	10 μM	Incubation	[24]
Human	Thimerosal	100–200 μM	Incubation	[4, 139, 151]
	Thimerosal	200 μM	Incubation	[57]

into the oocyte cytoplasm, by exposing the oocyte to a direct current (DC) pulse of electricity, also increases the concentration of intracellular-free calcium.

Even though the amplitude, duration and spatiotemporal dynamics of the artificially induced calcium transient increases are unlike those observed at fertilization, they are still able to initiate both the early and late events associated

with oocyte activation. In fact, many of the artificial activation treatments elicit only a single calcium rise, as opposed to the series of calcium oscillations induced by the fertilizing sperm.

Calcium ions

Measurement of the intracellular-free calcium concentration of oocytes with the fluorescent Ca²⁺ indicators, fura-2 or fluo-3, indicates that injection

of Ca^{2+} into the cytoplasm of unfertilized oocytes produces a single, short-lived calcium transient increase, or spike, in the mouse [53] and rabbit [32]. The duration and amplitude of such calcium spikes are dependent on the concentration of Ca^{2+} in the injected solution [32]. Investigators have successfully generated a series of calcium oscillations in mouse oocytes by applying a series of Ca^{2+} iontophoretic injections to unfertilized oocytes, but they were unable to mimic the calcium oscillations seen after injection of sperm extract or InsP_3 [53]. Surprisingly, however, a single injection of Ca^{2+} into the oocyte cytoplasm raises the intracellular-free calcium concentration sufficiently to trigger many of the events associated with normal oocyte activation. In porcine oocytes, a single injection of Ca^{2+} has been shown to induce cortical granule exocytosis, meiotic resumption, pronuclear formation, MPF inactivation, protein synthetic profile changes and parthenogenetic development to the blastocyst stage [119].

Another way of introducing Ca^{2+} into the oocyte cytoplasm is by making the oocyte plasma membrane permeable in the presence of Ca^{2+} with pulses of electricity. This method, often referred to as electrical activation, involves placing the oocytes between two electrodes, typically 0.5 to 1 mm apart, in a Ca^{2+} -containing, sugar-based non-electrolyte solution of mannitol, sucrose or glucose, and delivering one or more DC pulses of electricity across the electrodes. The sugar molecules destabilize the plasma membrane while the electrical stimulus causes pores to form in the lipid bilayer, which facilitates the uptake of extracellular calcium by the oocyte. The membrane remains permeable to extracellular calcium for several minutes, during which time the pores presumably reseal [122, 123].

A single electric pulse has been shown to induce a calcium transient increase in mouse [123], rabbit [124], cattle [122] and pig [5] oocytes. The amplitude and decay time of the calcium spike is affected by the electrical field strength, the duration of the pulse, the concentration of CaCl_2 in the pulsing medium and the pulsing medium itself [5, 123, 124]. A further source of variation in the oocyte uptake of extracellular calcium is the oocyte itself. The plasma membrane properties of the oocyte influence the formation of pores in the lipid bilayer and differ among species, change as the oocyte matures and ages, and are likely to be

affected by the conditions that the oocyte is exposed to.

An analysis of electrically activated pig oocytes using dynamic video imaging revealed that the calcium rise is always initiated in the region adjacent to the positive electrode and advances through the oocyte in the form of a wave at a velocity of 5 to 10 $\mu\text{m}/\text{sec}$ [5]. There are no subsequent calcium transient increases following the initial spike, as is seen at fertilization, unless additional electric pulses are applied [5]. These findings demonstrate that the calcium concentration change induced in oocytes by electric pulses is markedly different to that triggered by the fertilizing sperm. Despite this, the application of electric pulses to mammalian oocytes can induce both early and late events of oocyte activation.

Several investigators have demonstrated that cortical granule exocytosis, which is often referred to as the cortical reaction, is induced by electric pulses in porcine oocytes matured *in vitro* [5, 125–127]. Transmission electron micrographs show that the ultrastructural changes associated with cortical granule loss following electrical activation are similar to those observed in fertilized oocytes [5]. The levels of MPF activity in cattle [115, 120] and pig [33, 116, 117, 119, 128] oocyte extracts have also been found to decrease markedly following the application of electric pulses.

The formation of pronuclei is often used as an indicator of successful oocyte activation. Application of one or more electric pulses has been shown to induce meiotic resumption and pronuclear formation in mouse [123, 129], rabbit [8, 124], sheep [130], cattle [6, 131], pig [125, 126, 132–134] and marmoset [135] oocytes. Furthermore, electrically activated rabbit [8], cattle [6] and pig [126, 132, 133, 136–138] oocytes undergo cleavage and parthenogenetic development to the blastocyst stage.

Calcium ionophores

Calcium ionophores, such as A23187 and ionomycin, are compounds that are capable of forming a complex with a calcium ion and transporting it through a biological membrane by a carrier-type mechanism. Intracellular-free calcium determinations using fura-2 or fluo-3 have demonstrated that incubation of mature mammalian oocytes with A23187 or ionomycin generates a single calcium transient increase [3, 11,

12, 24, 139–142]. Mouse [140] and cattle [139] oocytes treated with 10 μ M A23187 experience an intracellular calcium rise that is of greater amplitude and of longer duration than the first rise induced at fertilization. The precise mechanism of the ionophore-induced calcium increase is not well understood, because in some studies extracellular calcium was required to produce a calcium rise in the oocytes, while in others a calcium transient increase was observed in oocytes treated in Ca^{2+} -free medium. The exact role of the internal calcium stores in the oocyte response to calcium ionophores is yet to be determined.

Treatment of oocytes with calcium ionophores also triggers the early and late events of oocyte activation. Cortical granule exocytosis [126, 127, 141–144] and zona pellucida modification, as assessed by the dissolution time of zonae in pronase and sperm penetrability [126, 141], have been observed in mammalian oocytes following treatment with A23187. In bovine oocytes, treatment with A23187 alone induces a decrease in the levels of MPF activity within 30 min [118]. Meiotic resumption and pronuclear formation have been observed in hamster [143, 144], cattle [131, 139] and pig [126, 132, 142, 145, 146] oocytes following treatment with A23187, with parthenogenetic development, as assessed by cleavage and blastocyst formation, also being reported [118, 126, 142, 145]. The concentration of A23187 and the duration of exposure to A23187 affect the efficiency of oocyte activation [131, 142]. In addition, the presence of extracellular calcium during treatment with A23187 increases the proportion of oocytes that develop parthenogenetically to the blastocyst stage [142].

Other studies have demonstrated that ionomycin induces the cortical reaction in rat oocytes [83, 84], MPF inactivation and meiotic resumption in cattle oocytes [121], and pronuclear formation in cattle [121] and sheep [130] oocytes. In response to ionomycin alone, bovine oocytes have also been shown to cleave and develop parthenogenetically to the blastocyst stage in an age-dependent manner [121].

Ethanol

Ethanol is thought to interact with cell membranes directly, polarizing the membrane and displacing Ca^{2+} from membrane phospholipids [147]. The addition of ethanol to the incubation

medium of mouse [140] and cattle [139] oocytes, caused a rapid increase of intracellular calcium that was greater and of longer total duration than the first increase observed at fertilization. The optimum concentration of ethanol found to stimulate the resumption of meiosis in bovine oocytes matured *in vitro* was determined to be 7 percent when an exposure time of 7 min was used [148]. Similar ethanol treatments induced pronuclear formation in pig [132], sheep [130] and marmoset [135] oocytes. Development of early cleavage stage parthenotes has also been demonstrated following exposure of cattle [118, 148, 149] and marmoset [135] oocytes to ethanol. There are no reports, however, of oocytes treated with ethanol alone developing parthenogenetically to the blastocyst stage. Oocytes activated with ethanol require an additional treatment with inhibitors of protein synthesis or protein phosphorylation to form blastocysts [118].

Thimerosal

The sulfhydryl reagent, thimerosal, oxidizes sulfhydryl groups on proteins, which causes calcium concentration changes in mammalian oocytes by sensitizing Ca^{2+} -induced Ca^{2+} -release from InsP_3 -regulated intracellular stores [17, 150]. Whereas the majority of oocyte activation treatments induce a single intracellular calcium increase, incubation with thimerosal generates repetitive calcium transient increases. Following the addition of 200 μ M thimerosal to incubation media of mouse [11], cattle [4, 151], pig [152] and human [57] oocytes, a series of intracellular calcium oscillations was induced in which the initial calcium transient increase was of greater amplitude than the subsequent spikes. Although thimerosal treatment alone has been shown to induce zona pellucida modifications as assessed by zona hardening [153], resumption of meiosis and parthenogenetic development have not been reported. Examination of oocytes immunocytochemically has revealed that exposure to thimerosal completely destroys the meiotic spindle, apparently by oxidizing tubulin thiol groups, which is known to disrupt tubulin polymerization [153]. However, the effects of thimerosal on the metaphase spindle of oocytes can be reversed by the sulfhydryl-reducing reagent dithiothreitol (DTT), presumably by reducing the oxidized tubulin thiol groups [152, 153]. Pig

oocytes sequentially incubated with thimerosal for 10 min and DTT for 30 min formed pronuclei and developed parthenogenetically to the blastocyst stage [152].

Inositol 1,4,5-trisphosphate receptor agonists

Agonists of the inositol 1,4,5-trisphosphate receptor (InsP₃R), which functions as a calcium channel, increase the intracellular-free calcium concentration of oocytes by gating calcium release from the endoplasmic reticulum. Numerous studies have demonstrated that the injection or electroporation of InsP₃ into the cytoplasm of mammalian oocytes induces an immediate, dose-dependent intracellular calcium increase [4, 11, 23, 25, 27, 34, 53, 139, 151, 154]. A single calcium spike is usually generated in response to InsP₃, however, repeated spikes have been observed in mouse [11, 53] and hamster [34] oocytes at the higher injected concentrations examined. Intracytoplasmic injection of InsP₃R agonists, such as InsP₃S₃ [151, 154], thapsigargin [12] and adenophostin [66], evokes similar increases in the intracellular-free calcium concentration of mammalian oocytes. Some of the events of oocyte activation have also been reported following injection of InsP₃R agonists, including zona pellucida modification, MPF inactivation, pronuclear formation and cleavage [24, 27, 29, 66].

Ryanodine receptor agonists

The ryanodine receptors (RyRs) are another major family of calcium channels that regulate the release of calcium from the endoplasmic reticulum in mammalian oocytes. Studies in mature mouse [12], cattle [23, 24, 27] and pig [25] oocytes have shown that a single calcium transient increase, similar in amplitude and duration to that induced by InsP₃R agonists, can be generated following the intracytoplasmic injection of RyR agonists, such as ryanodine, caffeine and cADPR. In response to injection with ryanodine, cattle oocytes have also been reported to form pronuclei and cleave [24].

Strontium ions

Like calcium, strontium in solution exists as a divalent cation, which, in muscle cells, can be taken up by the sarcoplasmic reticulum (SR) via the ATP-dependent transport system. In the presence of strontium, isolated SR have been shown to release calcium [3]. In Ca²⁺-free medium, mouse oocytes

incubated with 1 mM Sr²⁺ have been found to undergo a single intracellular calcium increase [155], while multiple calcium transients were generated in the presence of 4.6 mM Sr²⁺ [3]. Evidence indicates that the effectiveness of Sr²⁺ in inducing calcium changes and the associated events of oocyte activation is reduced in Ca²⁺-containing media [3, 156, 157]. It is thought that Sr²⁺ activates oocytes by displacing bound calcium in the oocyte, thereby triggering calcium-induced calcium release (CICR) [157]. In support of this, the reducing agent dithiothreitol (DTT) has been found to act synergistically with Sr²⁺ to initiate a series of calcium spikes in mouse oocytes [153]. One of the actions of DTT is to stimulate the entry of divalent cations into the oocyte, which leads to the onset of CICR [153].

G protein activators

Compounds that activate G proteins, such as serotonin, acetylcholine and GTP analogues, can also induce increases in the intracellular-free calcium concentration of mammalian oocytes. Activation of G proteins mediates the generation of InsP₃, which releases calcium from internal calcium stores. The results from studies on the effects of G protein activators on oocyte activation lend considerable support to the theory that sperm triggers the calcium oscillations seen at fertilization via a G protein-mediated pathway. Firstly, microinjection of G protein activators into mouse [153], hamster [34, 80, 158], rabbit [32] and cattle [151] oocytes elicits intracellular calcium oscillations similar to those seen at fertilization. Secondly, G-protein antagonists that have been shown to block the effects of the GTP analogue, guanosine-5'-O-(3'-thiotriphosphate) (GTP γ S), also inhibit fertilization [34, 80]. Finally, microinjection of GTP γ S into *in vitro* matured porcine oocytes has been shown to induce pronuclear formation and parthenogenetic development to the blastocyst stage [35, 119].

Protein synthesis inhibitors

Murine oocytes have been successfully activated by treatment with the protein synthesis inhibitor, cycloheximide [159]. Similar results have been obtained in human oocytes by incubation with puromycin, another protein synthesis inhibitor [160]. These compounds are thought to inhibit the synthesis of CSF, thereby reducing the level of MPF

activity in the oocyte, which triggers the resumption of meiosis [148]. In pig and cattle oocytes, cycloheximide treatment alone failed to induce activation as effectively as stimuli that generated an intracellular calcium rise [132, 148, 149]. However, when used in combination with ethanol or electric pulses, cycloheximide treatment increased the percentage of oocytes that formed pronuclei and cleaved [132, 148, 149, 161].

Protein kinase inhibitors

Inhibition of protein kinases with compounds such as H-7, staurosporine and olomoucine has been reported to induce the resumption of meiosis in mammalian oocytes [125, 162–165]. Protein kinase inhibitors are thought to suppress the level of MPF activity in oocytes directly, by blocking the phosphorylating activity of p34^{cdc2}, or indirectly, by inhibiting MAPK, which regulates p34^{cdc2} activity. In mouse oocytes, treatment with staurosporine resulted in the resumption of meiosis without the intracellular-free calcium concentration changing [165]. However, as with protein synthesis inhibitors, protein kinase inhibitors have been found to activate oocytes more effectively when combined with a calcium stimulus. For example, only very low rates of oocyte activation were obtained when unstimulated oocytes were treated with the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) [118]. In contrast, incubation with 6-DMAP following exposure of oocytes to either calcium ionophore or ethanol resulted in high rates of parthenogenetic cleavage and blastocyst formation [118, 130].

Activation of Nuclear Transfer Embryos

To date, a number of different activation treatments have been used in nuclear transfer (NT) procedures that resulted in the births of live cloned offspring. “Dolly” the sheep, the first animal cloned from a somatic cell, was produced by activating the reconstructed NT embryo with a series of electric pulses [166]. Since the report of this remarkable achievement, other investigators have described similar electrical activation protocols to produce somatic cell clones of sheep [167–169], cattle [170–172], goats [173] and pigs [174]. As well as being an effective activation stimulus, electric pulses mediate fusion of the

donor cell and the recipient cytoplasm. By applying the fusion pulses in Ca²⁺-containing media, the same stimulus can induce activation. Simultaneous fusion and activation is therefore a feature of many NT procedures. Cloned offspring have also been obtained by using electric pulses to activate recipient cytoplasts prior to NT [167, 175] and reconstructed embryos following injection of donor nuclei [176].

Sequential incubations with the calcium ionophore, ionomycin, and the protein kinase inhibitor, 6-DMAP, is another activation treatment that has been used to produce cloned sheep [130], cattle [177–179], pigs [180, 181] and goats [182]. Incubation with cycloheximide following exposure to ionomycin or electric pulses has also induced cattle [172, 183, 184], rhesus monkey [185] and mouflon [186] NT embryos to develop to term. Exposure to Sr²⁺, which induces multiple calcium transient increases in mouse oocytes, efficiently activated mouse NT embryos that yielded pups [187, 188]. In cattle, other treatments that have been used to successfully activate somatic NT embryos include incubation with 7% ethanol [189, 190] and cooling to 10 °C following oocyte aging [191].

Enucleated metaphase-II stage oocytes have been used as recipient cytoplasts in the vast majority of NT procedures described. Upon transfer to such a cell, the donor nucleus is exposed to high levels of MPF activity, which induces nuclear envelope breakdown and premature chromosome condensation (reviewed by [192]). Therefore, cells in the G₀ or G₁ phases of the cell cycle are typically isolated to supply donor nuclei that maintain a diploid chromosomal complement following transfer (reviewed by [193]). It has been suggested that nuclear remodelling and reprogramming may be facilitated by prolonged exposure of transferred nuclei to oocyte cytoplasmic factors. In cattle, NT embryo development *in vitro* was significantly increased by delaying activation for 4 to 6 h after fusion [179]. However, given that simultaneous fusion and activation treatments have been used to clone offspring in a number of species, it can be argued that such a delay is not critical for successful reprogramming. Enucleated metaphase-II oocytes that were activated prior to NT have also been used as recipient cytoplasts in some NT procedures. Donor cells that are in the S phase of the cell cycle maintain cell cycle synchrony with such a cell following transfer because the level of MPF activity

has declined. Due to the poor efficiencies of current cloning procedures, the effects of activation treatments on the developmental competence of NT embryos are poorly understood.

Strategies for Mimicking the Sperm-Induced Response

Investigators have adopted two strategies for mimicking sperm-induced responses observed at fertilization. One approach involves exposing the oocyte to multiple calcium stimulations in an attempt to duplicate the discrete intracellular calcium changes that are triggered by the fertilizing sperm. The other approach involves exposing the oocyte to a single calcium stimulus and a subsequent treatment that inhibits MPF activity.

Multiple calcium stimulations

Parthenogenetic studies have demonstrated that the developmental competence of oocytes may be increased by applying multiple calcium stimulations which induce calcium changes similar to those observed at fertilization [6, 8]. Oocytes exposed to electric pulse treatments that were repeated at intervals approximating the frequency of sperm-induced calcium spiking displayed an increased incidence of blastocyst formation compared with those exposed to a single stimulation [6, 138]. However, the further application of electrical pulses negated the beneficial effect that two or three stimulations imparted on pig [138] and cattle [6] oocytes, respectively. These findings suggest that the cumulative effect of the transient calcium increases was only beneficial below a threshold level, or that the application of too many electric pulses had a detrimental effect on some other aspect of oocyte viability, such as membrane integrity. The former proposal supports the theory that excessive calcium is detrimental to the function of cells [194].

Several studies have shown that the high level of active MPF, which maintains meiotic arrest in M-II oocytes, is reduced following artificial activation [115, 128, 195, 196]. In sperm-penetrated oocytes, the level of active MPF stays lower than that at M-II for at least 16 h [115, 128, 197]. Collas *et al.* [115] reported that the MPF activity in electrically activated bovine oocytes was depleted further and suppressed for longer when multiple stimulations

were administered compared with a single stimulation. This finding suggests that the first calcium increase initiated MPF inactivation and that the subsequent calcium increases maintained the suppression of MPF activity.

Coupling a calcium stimulus with a treatment that inhibits MPF activity

The other strategy adopted by many NT researchers is to couple a single calcium stimulus with a treatment that inhibits MPF activity [149, 198]. As already mentioned, such combined treatments have induced NT embryos to develop to term in a number of species. Cycloheximide has been used to inhibit MPF activity in electrically activated cattle [172, 183, 184], goat [199] and rhesus monkey [185] NT embryos and calcium ionophore-treated mouflon NT embryos [186]. Similarly, 6-DMAP has been used to suppress MPF activity in calcium ionophore-treated NT embryos of sheep [130], cattle [177–179], pigs [180, 181] and goats [182].

In porcine oocytes, a combined electrical activation and 6-DMAP treatment hastened the suppression of MPF activity compared with electrical activation alone [128]. Furthermore, MPF reactivation was delayed by 6-DMAP treatment such that the kinetics of MPF activity were similar to that of inseminated oocytes, which correlated with an increased incidence of parthenogenetic blastocyst formation [128]. Instead of attempting to mimic the calcium oscillations induced by the fertilizing sperm, this approach endeavours to duplicate a critical cell cycle event downstream of the initial sperm-induced response. However, 6-DMAP is a broad-spectrum protein kinase inhibitor that may effect the phosphorylation state of proteins other than the catalytic subunit of MPF—proteins that may be involved in the remodelling or reprogramming of donor nuclei. Therefore, it may be preferable to regulate MPF activity in NT embryos by using reagents that more specifically inhibit p34^{cdc2}. There are a growing number of kinase inhibitors, including roscovitine, olomoucine and bohemine, which act by competing for the ATP-binding domain of cyclin-dependent protein kinases. Recently, specific inhibitors of cyclin-dependent protein kinases have been used in conjunction with a calcium-releasing stimulus to induce oocyte activation in cattle [200] and rhesus monkey oocytes [201].

Conclusions

Artificial activation treatments are unable to precisely duplicate the pattern of calcium oscillations that are induced by the fertilizing sperm. Although a single calcium stimulus induces many of the cellular events associated with oocyte activation, the application of multiple calcium stimulations may increase the developmental competence of the oocyte compared with a single stimulus. Evidence suggests that maternal machinery involved in the activation response is triggered by the sperm activating signal but not by artificially induced calcium-release. Isolation of the

sperm cytosolic factor would undoubtedly enhance our understanding of oocyte activation and facilitate the development of improved artificial activation treatments. It is well established that meiotic resumption requires the inactivation of MPF. Premature reactivation of MPF following the application of a "weak" calcium-increasing stimulus may result in aberrant cell cycle progression. Currently, the best strategy for artificially mimicking the sperm-induced activation response in NT embryos of the domestic species may be to couple a single calcium stimulus with a treatment that suppresses MPF activity.

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